

Detection of microbial food contaminants and their products by capillary electromigration techniques

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Running title: Analysis of microbial food contamination by CE.

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Abbreviations: **AFLP**, amplified fragment length polymorphism; **AGE**, agarose gel electrophoresis, **ASP**, amnesic shellfish poisoning; **BGE**, background electrolyte; **CFP**, ciguatera fish poisoning; **DA**, domoic acid; **DCIP**, 2,6-dichlorophenolindophenol; **DSP**, diarrhetic shellfish poisoning; **DTX**, dinophysistoxin; **ERIC**, enterobacterial repetitive intergenic consensus; **FB1**, Fumonisin type 1; **GTX**, gonyautoxins; **MC**, microcystins; **MLVA**, multiple locus variable-number tandem-repeat; **MTX**, maitotoxin; **NEO**, neosaxitoxin; **NSP**, neurotoxic shellfish poisoning; **OA**, ochratoxin A; **OkA**, okadaic acid; **PSP**, paralytic shellfish poisoning; **PEO**, polyethylenoxide; **STX**, saxitoxin; **TMV**, tobacco mosaic virus; **T-RFLP**, terminal restriction fragment length polymorphism; **TTX**, tetrodotoxin; **UTIs**, urinary tract infections.

Keywords: CE, food, microorganisms, foodborne pathogens, toxins, review.

ABSTRACT

This work reviews the different analytical strategies based on capillary electromigration techniques developed for detecting microbial contaminants and their products in food matrices. Namely, this work presents an exhaustive and critical review, including works published till March 2007, on capillary electrophoresis (CE) methods developed to detect and identify contaminants of microbial origin that represent a hazard to humans in foods. First, an overview on the strategies adopted for the analysis of intact microorganisms is presented. Next, CE methodologies based on the analysis of microbial constituents, including those based on Genomics and Proteomics approaches, are described. Finally, CE methods developed for detecting microbial toxins are discussed.

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1. Introduction

Foodborne illness, commonly known as food poisoning, occurs when food becomes contaminated with harmful species. Although pesticides and metabolites can give rise to important health problems, the vast majority of food poisonings are the direct result of microbiological hazards induced by bacteria, viruses, parasites, toxigenic molds or microalgae. Among them, pathogenic (disease causing) bacteria are the main source of foodborne illness. Besides the health problem, the presence of pathogenic foodborne organisms and their toxins can also give rise to significant economic losses to food industry and public health system. In 2000, the Economic Research Services (USDA) estimated the annual US economic cost equal to \$6.9 billions due to only the five major bacterial foodborne pathogens (United States Department of Agriculture, <http://www.ers.usda.gov/briefing/FoodborneDisease/features.htm>; checked on March 2007).

Most microbial foodborne pathogens are capable of growing undetected in foods because they do not produce off-flavor, color, or texture changes. Therefore, identifying, monitoring, and quantitating such microorganisms and their toxic products are of paramount importance. Generally, methods for the detection of pathogens in foods need to meet a number of minimum requirements: (a) they must be fast because of the perishable character of certain foodstuffs and due to the rapidity needed for food production and distribution, (b) they must be selective since pathogens may be present as a small fraction of the innocuous microbial population present in food, and (c) they must be sensitive because an infectious dose may be as low as a single pathogenic cell.

The examination of foods for the presence of microorganisms and their toxic products is challenging due to foodstuffs come in many physical formats and their composition is even more

diverse as they are made up of various combinations of ingredients resulting in very heterogeneous samples. Moreover, microorganisms and toxins are usually not uniformly distributed in foods and an aliquot tested may not necessarily be representative of the overall sample. To overcome these problems, food industry and regulatory agencies have traditionally used conventional microbiological methods based on well established microbiological techniques [1]. These procedures involve a series of culture-based pre-enrichments to increase the number of pathogens while minimizing the growth of the innocuous microbial flora. After enrichment, samples are grown on selective media in order to isolate individual bacterial colonies. Additional biochemical or immunological characterization of individual bacterial clones is usually necessary to identify the pathogen [2]. Similarly, in microbial toxin testing, extraction and concentration steps are used prior to detection by serological or animal assays [3]. Despite these methods have been accepted for decades, they are extremely labor intensive, the interpretation of results is often subjective, and for some pathogens the total test time can be of several days. Consequently, these classical methods need to be improved in order to quickly test the microbiological quality and safety of foods. Besides, it has to be highlighted the few alternative methods available to analyze entire microorganisms.

In addition to the aforementioned problems with conventional microbiological methods, there is a lack of suitable growth media to support the growth of certain species, known as viable but nonculturable (VBNC) organisms. As a result, some foodborne pathogenic microorganisms, that under certain conditions enter the VBNC state, such as certain strains of *Salmonella*, *Shigella*, *Vibrio cholerae*, and enteropathogenic *E. coli*, are a serious threat to food safety and health [4].

Reduced labor requirements and shorter analysis times can be achieved by adopting alternative analytical methods. The availability of novel technologies, including those developed for the production of specific antibodies, amplification of specific deoxyribonucleic acid (DNA) target sequences, as well as the improved quality of commercial specific substrates, including labeling and staining reagents, have already made possible the development of faster microbiological tests in foods [5]. Nevertheless, owing to our current inability to detect minute signals generated by microorganisms amid the noise existing in most food matrices, some culture enrichment as well as sample extraction and purification procedures are still required prior to their analysis, which on the other hand, compromises the time of analysis. Besides, positive results obtained by these methods are often considered as tentative and require further confirmation [6].

In recent years, the above-mentioned limitations have encouraged the development of new methodologies as for example biosensors, capable of detecting the presence of microorganisms, and their toxic metabolites rapidly and accurately [6-9]. Of particular interest are the remarkable improvements in the development and performance of instrumental methods such as flow cytometry, matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), electrospray ionization mass spectrometry (ESI-MS) or DNA-microarrays, among others, that may have a tremendous impact on microbial food analysis [10-12]. However, the strict conditions to perform the analysis and the requirement of expensive and unique instrumentation restricts their application to routine food inspection.

Among these instrumental techniques, capillary electrophoresis (CE) may represent an interesting alternative to the current microbiological methods for the determination of microbial contaminants

in foods. CE has emerged as a separation technique that provides fast and efficient separations with minimum consumption of sample and reagents, allowing automation. In food analysis, CE has sufficiently demonstrated its potential and versatility for solving analytical problems such as ensuring compliance with food and trade laws, adulteration, contamination, quality control, chemical composition of foods, etc [13-20]. It is noteworthy to mention the increasingly growing interest on the application of CE to the microbiological research field that is reflected by the high number of works published on this topic during the last decade.

The following sections of this review paper describe the various approaches most recently taken (including works published until March 2007) to detect and identify contaminants of microbial origin that represent a hazard to humans by CE techniques. First, a brief overview on the strategies adopted for the analysis of intact microorganisms with particular emphasis on reported food applications is presented. Next, CE methodologies based on the analysis of microbial constituents, including those based on Genomics and Proteomics approaches, for microbiological food testing are described. Finally, CE methods developed for microbial toxin detection are discussed. A summary of some representative applications of CE in this area is given in Table 1

2. CE separations of intact microorganisms.

Owing to their size, within the sub-micrometer and micrometer range, microorganisms are biological assemblies that can be characterized as charged colloids. The charged surface of microorganisms usually originates as a consequence of two events: (a) the ionization of acidic or basic molecules represented by amino acid residues, organophosphates, acidic carbohydrate groups

and sulfates moieties, among others; and (b) the adsorption of ions from the surrounding solution to the surface of the cell [21]. Therefore, altering the composition and pH of the surrounding solution will affect the electrophoretic mobility of microorganisms [22]. For a more detailed treatment of these concepts, including the theoretical considerations in the study of the colloidal features of microorganisms by CE, interested readers can take resort of some works published on this topic [21-25].

CE experiments with intact microorganisms were first reported by Hjertén *et al.* [26] who showed that *Lactobacillus casei* bacteria and Tobacco Mosaic Virus (TMV) would migrate in solution under the effect of an electric field. These experiments were extended to demonstrate the quantitative dependence of electrophoretic mobility of cylindrically shaped TMV particles with translational frictional coefficient due to variations in the alignment of the virus with the electric field [27]. Later, Ebersole and McCormick [28] separated mixtures containing various bacterial species, including among others, the foodborne enteropathogen *Staphylococcus aureus* and the microbial indicator of fecal contamination *Enterococcus faecalis*. Large capillaries (250 cm x 100 µm id) were used and recovery studies demonstrated that more than 90% of the bacteria remained viable after 70 minutes under 120 V/cm. Using similar conditions, Pfetsch and Welsch [29] also analyzed by CE a mixture of three different bacteria from the genera *Pseudomonas* and *Alcaligenes*. In their publication [29], authors reported that separations performed under 120 V/cm using a background electrolyte (BGE) with an ionic strength ranging from 0.2-15 mM did not damage the microbial cells under study. One year later, Glynn *et al.* [30] observed different mobilities (referred to as multimodal mobilities) for the same strain in the analysis of environmental samples by CE. This fact was attributed to heterogeneities in the charge density of the microbe surface [30,31]. CE

separations of several bacteria and fungi in free solution were also explored by Torimura and co-workers [32]. For each microorganism, electrophoregrams showed one main broad peak representing well-dispersed cells, which was reproducible in time and shape, and several non-reproducible spike-like peaks representing cell aggregates. The addition of glucose and the electron acceptor 2,6-dichlorophenolindophenol (DCIP) to the separation buffer served to measure enzymatic activities during their CE separation. A shift in the baseline of the electrophoregram obtained using 605 nm as detection wavelength was indicative of a decrease in the DCIP concentration in the running buffer as a consequence of the reducing activity of *E. coli*. Later on, Yamada *et al.* [33] separated a mixed culture of two microbial cell strains, *Cellulomonas cartae* KYM-7 and *Agrobacterium tumefaciens* KYM-8, by capillary gel electrophoresis (CGE) and capillary zone electrophoresis (CZE). Results obtained in recovery experiments demonstrated that, despite of the broader peaks obtained by CGE, it provided peaks slightly more pure than CZE (98% vs. 90% of purity).

The common limitations for the aforementioned studies are low sensitivity (generally, limits of detection were within the range of 10^3 - 10^4 cells injected) and large peak width, often wider than one minute, which was typically interpreted as a consequence of the inherent electrophoretic heterogeneity in bacterial populations. This later observation restricts the use of these methods for microbial analysis since relatively large differences in mobility would be required for complete separations.

On the other hand, by the time the two latter papers mentioned [32, 33] came to light, Armstrong and co-workers had published a novel manner of separating microorganisms in exceptional sharp

peaks [34]. In various reports, authors demonstrated that the use of small amounts of polyethylenoxide (PEO, i.e. 0.0125% of polymer) into a BGE containing tris(hydroxymethyl)aminomethane (Tris), borate and ethylenediaminetetraacetic acid (EDTA; TBE buffers) was essential to achieve high efficiencies (often exceeding 10^6 theoretical plates/m). Although critically controlled buffer and sample preparation steps were required to keep the cells intact and the separation reproducible [34], variations in the migration time of microorganisms were sometimes observed among samples. This effect was associated to the different ionic strength of the analyzed samples [35].

In a separate report, Armstrong's group [36] carried out the separation and characterization of cell aggregates using the aforementioned polymer-based BGE in order to gain some insights about the tendency to self-aggregate that microorganisms exhibit in aqueous solutions. They demonstrated that, previous sonication treatment of the sample to increase cell dispersion, the separation method was able to distinguish between *Micrococcus luteus* dispersed cells from cell clusters of the same strain that exhibited a different aggregational degree. Similar results were also obtained for the yeast *Saccharomyces cerevisiae*. However, in contrast to these microorganisms, *Alcaligenes faecalis* cells could not be efficiently dispersed by sonication, indicating a stronger association among bacterial cells. Experiments were extended to identify and quantitate active bacteria in dietary supplements and other consumer products, containing *Bifidobacterium infantis* and *Lactobacillus acidophilus* by diluted polymer-based BGEs and laser induced fluorescence (LIF) detection [37]. Cell viability was assayed by selective staining of injured cells using propidium iodide, and the non-selective staining with SYTO-9, which stains all bacteria in a population. Green and red fluorescent light were monitored simultaneously with a LIF detector (λ_{ex} 488 nm) equipped with

appropriate filters. The ratio of both peak areas obtained from solutions containing different proportion of dead cells served to construct a standard curve ($R^2=0.995$), allowing the rapid and accurate quantitation of bacteria in real samples. The versatility of this technique for viability assays of different types of cells was later demonstrated in two different papers [38, 39].

Further studies by the same group were focused on the study of mechanistic aspects involved in the generation of such remarkably sharp peaks by diluted polymer-based BGEs [40-42]. By using a charge-coupled device (CCD) camera coupled with laser induced fluorescence, it could be observed that a stacking of the injected sample band and a decrease in the normal band broadening were responsible for the extremely narrow bandwidth of these peaks. Authors associated this phenomenon with the presence of the dilute polymer in the separation buffer, the effect of the electric field, and the EOF observed countercurrent to the microbial cell mobility [40]. Moreover, a number of variables including type, molecular weight and concentration of polymer, pH, and injection time, required optimization for efficient and reproducible CE separations [41]. Furthermore, Zhen and Yeung [43] investigated a possible mechanism to explain such microbial aggregation observed during CE with dilute polymer solutions. Evidences suggested that microbes move at different directions and velocities under the conditions assayed. This motion increases the possibilities of collision between microbes and aggregation. Results obtained from this study indicated a strong influence of the ionic strength as well as the type of buffer on cells aggregation. PEO was believed to decrease EOF, to adsorb onto the cell surface acting as a bridge between two microbes, and also to act as a coating around the cell surface, preventing borate and EDTA complexation and, therefore, increasing the probability of aggregation [43].

In successive years, several laboratories directed their studies toward the analysis of microorganisms with interest in food industry, clinical diagnosis and environmental control, using diluted polymer-based BGEs [44-50]. Shintani *et al.* [44] successfully detected *Salmonella enteritidis*, the causative agent of a major foodborne disease, by CE and the aid of UV and LIF detectors. Thus, separation and fluorescence detection of mixtures containing *S. enteritidis* and *S. typhimurium* were carried out using TBE buffer containing 0.01% sodium alginate, 0.1% sodium chloride, and SYTO-9 as additives [44]. Good linear relationship ($R^2=0.9992$) was observed between the detected fluorescence peak area and cell counts in a range of 3-114 injected *S. enteritidis* cells. Moreover, the relative standard deviation (RSD; n=3) values were below 0.4% and 6% for migration times and peak areas, respectively. The applicability of the CE method for food analysis was also improved by adding a fluorescent-labeled polyclonal antibody to the separation buffer for the selective identification of *Salmonella* serotypes within a mixture containing non-*Salmonella* strains [44]. Moon and Kim [45] showed the potential of diluted PEO-based BGEs for the characterization of probiotic-containing products. Nevertheless, the reproducibility of the CE patterns obtained from the analysis of microorganisms by this strategy seems to be largely affected by variables such as BGE ionic strength, as well as by pretreatment, physiological state and storage conditions of cells [46]. In addition, Hoerr *et al.* [47] reported that such susceptibility of the separation system to small experimental conditions variations also depends on the microorganisms under study. The diluted PEO-based BGE also proved to be invaluable for the rapid identification and quantitation of *Edwardsiella tarda*, a pathogen of commercially important fish [48]. In this case, authors used a blue-LED pulse light source at 473 nm to induce fluorescence of bacterial cells containing nucleic acids stained with SYTO-13. The recovery of spiked *E. tarda* cells in fish samples was relatively low (70%), which was explained as a consequence of fluorescence

quenching by the matrix or possible cell lysis. Later, the detection of the foodborne enteropathogenic *E. coli* and *Pseudomonas aeruginosa* was also investigated by the same group using this CE approach but with a relatively higher buffer concentration (150 mM) [49]. At such buffer concentration, the mobility of the microorganisms decreased but the bacterial cells resisted decomposition and therefore, no extra significant noise or unwanted peaks were detected in the electrophoregrams. More recently, Klodzinska *et al.* [50] have investigated the use of diluted PEO-based BGEs for the rapid identification of *E. coli* and the emerging pathogen *Helicobacter pylori* in complex samples.

Buszewski *et al.* [51,52] investigated microbial separations in modified CE capillaries. A first attempt involved the use of acrylamide-modified capillary and UV detection to determine the electrophoretic mobilities of *E. coli*, *Pseudomonas fluorescens*, *Bacillus cereus* and *Proteus vulgaris*. Under normal polarity and injecting a microbe mixture into the shorter part of the capillary, it was possible to separate the microorganisms in seven minutes [51]. The same group also separated a mixture of other five bacteria using trimethylchlorosilane- and divinylbenzene-modified capillaries [52].

The combined use of fluorescent labeled antibodies with CE-LIF seems to be promising for the analysis of foodborne pathogenic bacteria in complex samples. Specific antibodies can be used to selectively capture, label and detect antigens associated with the target microorganism. Kourkine *et al.* [53] investigated the use of fluorescent labelled antibodies to purify and stain *E. coli* and *Salmonella* from contaminated meat samples prior CE-LIF by two different strategies. Namely, a direct approach involving the detection of fluorescent labelled bacteria, and an indirect approach

involving detection of fluorescent antibodies captured and then, released by the target bacteria upon sonication in presence of a surfactant (Tween). In both strategies, a fused-silica capillary dynamically coated with poly-N-hydroxyethylacrylamide was used to prevent protein adsorption onto the capillary wall. The direct approach provided broad peaks and interferences of the bacteria with free antibodies that could be minimized introducing several washing steps of the sample prior CZE analysis. This method was successfully used to detect 100-200 colony forming units (cfu) of *E. coli* in 25 g of contaminated meat in 6-8 h (including 5 h of enrichment). On the other hand, the indirect approach provided better sensitivity but the quantitative capabilities were diminished due to the variability in the amount of antibodies released upon sonication.

Recently, Gao *et al.* [54] developed a highly specific CZE-UV method for the detection of *S. aureus* in complex samples. The method involved the use of antibody-coated latex particles in the BGE able to bind *S. aureus* cells during the separation. The complex bacterium-latex particle had a different electrophoretic mobility from the corresponding free *S. aureus* cells and therefore, was detected as a single peak in the electrophoregram. A limit of detection (LOD) of 9.0×10^5 cfu per mL was reported and authors indicated that the sensitivity of the proposed method would be sufficient to detect a contaminated food sample with *S. aureus* at typical poisoning levels.

As mentioned above, some properties of the surrounding solution (pH, ionic strength and ionic composition), as well as the size and nature (surface chemistry) of each biocolloidal particle can control the type of ion and amount that can be adsorbed onto its surface. This latter aspect has been examined for the identification and quantitation of bacterial contamination in food samples by CE-UV. Thus, eight bacteria species of interest to food safety including *Yersinia enterocolitica*,

Leuconostoc mesenteroides, *S. enteritidis*, *Listeria monocytogenes*, *E. coli*, *Lactobacillus plantarum*, *S. aureus* and *Enterococcus faecium*, were effectively resolved using a bare-fused silica capillary (peaks 1-8 in Figure 1, respectively). The effect of adding cations and anions to the separation buffer (based on a 25 mM phosphate BGE at pH 7.0) on the resolution was investigated. Among all the ions included, only the presence of calcium and myoinositol hexakisphosphate showed a beneficial effect on resolution and peak shape. Moreover, the combination of both ions in the separation buffer demonstrated to have a synergistic effect improving peak width and shape. These authors proposed a sample treatment for the analysis of solid and liquid food samples prior CE-UV analysis [55]. The procedure consisted of centrifuging the sample suspension at low velocity and filtering in two steps using different pore sizes to retain bacteria. Filters were finally washed and the retained bacteria were re-suspended in separation buffer for their analysis. Using this procedure, recoveries ranged from 87 to 120% with a reported precision between 3.3 and 7.7%. The applicability of the proposed method to food analysis was demonstrated by analyzing spiked corn flakes and juice samples with 10 cfu/mL (or mg). Although the sample treatment was useful to remove interfering compounds, owing to the lack of sensitivity of the method (LOD ~ 10^8 cfu/mL), an enrichment step of 7 h was necessary for the detection of bacteria.

Improvements in sensitivity of microbial CE separations have been recently reported. Horká *et al.* [56] used a fluorescent amphiphilic additive (pyrenebutanoate) into the separation buffer to modify the surface of microorganisms providing high separation efficiencies and good reproducibility (RSD < 2% for migration times). The fluorescence signal of the additive adsorbed onto the bacteria surface afforded the detection of less than ten injected cells (see Figure 2).

Microbial contamination testing often requires ultrahigh sensitivity as for instance for sterility tests whose detection limit must be a single cell. A recent CE method developed to meet this criterion involved the injection of the sample containing the microorganisms followed by the injections of two plugs, the first containing BGE, and the second containing a “blocking agent” [57, 58]. The BGE contained dilute cetyltrimethylammonium bromide (CTAB) that, under reversed polarity, swept microbial cells out of the sample zone toward the cathode. After that, the cells encountered the “blocking agent” that eliminated their mobility and induced aggregation [57]. The microbial cells were stained with a fluorescent dye prior injection for sensitive detection by LIF. Several factors affect method sensitivity, particularly, composition of the “blocking agent” [58]. To illustrate the sensitivity achieved, Figure 3 shows the electrophoregrams of single cells of various bacteria and fungi detected by using this method.

Nowadays, one of the major challenges in this area is the analysis of large volumes of sample containing low number of microbes in highly interfering matrices. Removal of microorganisms from representative food samples into clean suspensions provides volumes of about 100 mL that often require amplification of microbial signals to detectable levels. Such analyte amplification is mostly performed by overnight enrichment in growth media, making these new methods unlikely candidates for direct determinations since enrichment makes difficult any quantitative approach. Accordingly, the adoption of strategies aimed to reduce the suspension volume while keeping microbial cells intact prior to their CE analysis may be helpful as it has been demonstrated with other instrumental approaches [59-62].

3. Analysis of microbial constituents by CE.

Conventional phenotypic analysis used for microorganisms identification requires intense labor and long times of analysis. Besides, results are often not conclusive since many of the observed physiological or biochemical characteristics are not restricted to a single microorganism species. Innovative techniques based on the analysis of microbial constituents have been employed to alleviate the lack of speed and accuracy in microbiological identification. Owing to the advent of the *Genomics* and *Proteomics* era, the development of such techniques has seen a huge number of applications in food microbiology.

3.1. Genome-based methods.

Nucleic acids are ideal molecules for the specific detection of microorganisms in foods. The sequence of DNA provides highly specific biological information at every taxonomic level. Techniques that allow the rapid amplification and detection of nucleic acid molecules have been the key for the remarkable popularity and success that molecular methods have gained during the last years in food analysis. Particularly, the development of polymerase chain reaction (PCR) as an analytical tool has been fundamental in this field. PCR methods theoretically would bypass the need for culturing organisms since all that is required is genomic DNA isolated from the organisms under investigation to perform their analyses. Nevertheless, numerous attempts to use PCR in food analysis have found that many foods contained substances that inhibited or interfered with PCR, leading to false negatives. As a result, some cultural enrichment is still a requirement prior to PCR [63]. Another shortcoming of DNA amplification-based methods accounts for the necessity of sensitively detecting the presence of the target sequence (amplicon) after the amplification. This final step has traditionally been performed by agarose gel electrophoresis (AGE). Besides the insufficient resolution and sensitivity of AGE, the use of carcinogenic substances and the need to

visualize the amplicons were not user-friendly enough for adequate diagnostic setting [64]. To overcome such limitations, novel CGE methods have been combined with PCR-based methods to successfully detect microbial food contaminants with high sensitivity and specificity. As an example, PCR followed by CE were used to detect *L. monocytogenes*, a potentially lethal foodborne pathogen. This pathogen is often present in a wide variety of products and has the potential to grow under proper refrigerated storage. Detection of *L. monocytogenes* in foods is complicated by the presence of other closely-related but non-pathogenic species. *L. monocytogenes* strain identification was reported based on enterobacterial repetitive intergenic consensus (ERIC) fingerprints resolved by CGE-UV with adequate sensitivity, resolution and accuracy of DNA sizing [65]. Scicchitano and Hirshfield [66] also developed a PCR-CGE method for the sensitive detection of *Clostridium botulinum* type E neurotoxin gene in smoked fish.

For terminal restriction fragment length polymorphism (T-RFLP)/CGE-LIF analysis, the locus of interest is amplified by PCR with gene-specific labeled primers; therefore, previous knowledge of the DNA sequence is a requirement. Amplicons are then subjected to digestion with an appropriate restriction endonuclease followed by separation of the DNA fragments by CGE-LIF. This method was applied to analyze pathogens in fish. Using the fluorescent dyes HEX and 6-FAM to label the primer pair at the 5'-end, the CGE-LIF method demonstrated to be useful to detect as low as 30 cfu of *Flavobacterium psychrophilum* per mg of tissue [67].

Amplified fragment length polymorphism (AFLP)/CGE analysis is based on the selective amplification of a subset of DNA fragments generated by digestion of genomic DNA with two restriction enzymes. Specific oligonucleotide adapters are then ligated to these restriction sites to

create a template sequence for subsequent high stringency PCR amplifications. Adapters used for amplification were originally radioactively labeled for subsequent analysis by means of conventional sequencing gels, but the use of CGE-LIF and fluorescent-labeled primers greatly simplifies the procedure. The genotyping of *Salmonella enterica* and *Campylobacter jejuni* has been successfully performed using this methodology [68, 69].

Enterohemorrhagic *E. coli* O157:H7 is the causative agent of a gastrointestinal syndrome. This microorganism produces two versions of verotoxin (VT1 and VT2) with similar activities that interfere with the protein-synthesis process in the cell. The sensitive and reproducible detection of VT1 and VT2 toxin genes was achieved by the combination of three molecular techniques (allele-specific PCR, single strand conformation polymorphism and cleavase fragment length polymorphism) followed by CGE [70].

The aforementioned PCR-based methods were designed for the detection of a single microorganism species in one analysis. On the other hand, with multiplex PCR more than one target sequence can be amplified in a single reaction allowing the sensitive detection of several pathogens in a single analysis. However, for a successful multiplex PCR assay it is important to optimize the reaction parameters to avoid the formation of spurious amplification products and uneven amplification of target sequences [71]. This novel concept of PCR was exploited in combination with CGE-LIF to achieve simultaneous detection of several bacteria in a single analysis [72,73]. Multiplex PCR reduced the time required for sensitive detection of *Salmonella*, *L. monocytogenes* and *S. aureus* in artificially inoculated raw beef samples since LODs ranging from 5.7×10^2 to 2.6×10^3 cfu/mL were achieved in a single assay without any enrichment. Further improvements in sensitivity could

be accomplished with additional pre-enrichment for 6 h which afforded the detection of 57, 79, and 260 cfu/mL of *Salmonella*, *L. monocytogenes* and *S. aureus*, respectively [72] (see Figure 4). A similar strategy was used to differentiate several food-spoilage bacteria of interest in food industry. The genera *Leuconostoc* and *Carnobacterium*, the nonmotile group of species within the genus *Carnobacterium*, and the three species of the group individually (*C. divergens*, *C. gallinarum*, and *C. maltaromicum*) were identified by multiplex PCR/GCE-LIF. In both works [72,73], the use of CGE-LIF to analyze the PCR products was significantly beneficial in terms of resolution and sensitivity when it was compared to AGE, as can also be seen in the example given in Figure 4.

Other approach to identify foodborne pathogens is the combined use of multiple locus variable-number tandem-repeat (MLVA) with CGE-LIF. MLVA is based on the amplification of short nucleotide sequences that are repeated multiple times in the bacterial genome. These short sequences often vary in copy number in different strains of a species, thus providing a means of discriminating between strains. Using labeled primers with different dyes, it is possible to perform multiplex PCR followed by CGE-LIF to provide microbiological identification with less uncertainty than with AGE analysis. This methodology has proven to be a powerful tool for differentiating serovars of *S. enterica* and Shiga toxin-producing *E. coli* O157, and therefore, it may be a helpful tool in foodborne disease surveillance programs [74, 75].

A common limitation of PCR-based methods is the presence of false positives since a peak could be due to a non-specific reaction product with a similar size to that of the expected product. As the risk of false positives should be avoided, additional confirmation methods can be developed in order to discriminate specific from unspecific amplicons. The most reliable confirmation method is

amplicon sequencing, but it is very expensive, and its use is generally restricted to optimization steps. Also, e.g., hybridization with specific probes would prove that the expected amplicon is obtained.

3.2. Protein biomarkers.

Pathogen identification based on detection of protein biomarkers by mass spectrometry is also growing [11,76,77]. MALDI-MS and ESI-MS techniques have been employed to obtain protein/peptide profiles representative of a particular microorganism and then, to compare them with the profiles obtained from unknown bacterial samples to identify them. The success of this approach for microbiological identification lies on the ability to obtain reproducible MS profiles from particular microorganisms. Also, the application of this approach to food analysis presents some difficulties due to inherent variability of cellular composition of bacteria under different environmental conditions. To overcome the problem of poor identification induced by limited mass accuracy in the determination of relative molecular mass of proteins when using low resolution MS analyzers, Hu *et al.* [78, 79] applied CE-MS/MS to selected peptide ions to obtain partial sequences of protein biomarkers. The coupling CE-MS/MS can provide a highly selective and sensitive analytical method for characterizing the analytes present in complex mixtures. Thus, preliminary analysis by CE-MS/MS of proteolytic digests of pure bacteria extracts and subsequent database searches were carried out to select abundant marker peptide ions that were specific to the bacterial species of interest. In part, the applicability of this type of approaches to the identification of a wide number of microorganisms depends on the availability of gene sequence databases. Up to date, the complete genome of more than 35 microorganisms has been sequenced and comprehensive studies

at the Proteome level have been recently published [80]. However, it is clear that there is a big room here for development of new CE and CE-MS based methods.

4. Analysis of microbial toxins.

Due to the dangerous nature of microbial toxins to human health, the occurrence of these substances in food represents a serious concern worldwide. As a consequence, many countries have set maximum acceptable levels of certain toxins in foods. Moreover, as new toxins are being identified as well as the potential risk that they represent to public health, the imposition of regulatory limits for toxins in foods is likely to continue. Accordingly, there is a demand for reliable methods that can provide appropriate analysis to enforce legislative limits at acceptable cost and time.

4.1. Mycotoxins.

Mycotoxins are low-molecular weight, non-proteinaceous compounds produced by filamentous fungi that can enter the human food chain by a large number of products. There is a significant structural variability between the different mycotoxins and depending on the type and intake level, mycotoxins are capable of producing different responses. The diversity of sample matrices where mycotoxins can be found, their presence at trace levels in complex matrices, and their non uniform distribution in raw materials are factors that make the analysis of mycotoxins challenging. A number of techniques have been used for mycotoxin analysis, but high-resolution separation techniques are the preferred methods since they provide the sensitivity, accuracy and precision required for trace analysis. Methods based on HPLC or GC, sometimes coupled to MS detection, have demonstrated good performance in the analysis of mycotoxins in different matrices [81]. On the other hand, rapid methods based on enzyme-linked immunosorbent assay (ELISA) with plate

reader for optical density measurement have been recently developed, but special attention must be paid to possible matrix interference problems and cross-reactions [81]. Although these methods have been shown to provide good results, elaborate sample processing step for extraction and clean-up is essential to obtain detectable levels of mycotoxins in food commodities.

Micellar electrokinetic chromatography (MEKC) and CZE in combination with different detection schemes have been the main CE modes applied for mycotoxin analysis. While the development of MEKC methods has been devoted to detect mostly aflatoxins, CZE methods have been developed for the detection of fumonisins, ochratoxins and moniliformin. Regarding the detection techniques, laser-based fluorescence is still the most often adopted. However, MS and UV detectors, often with recording of absorption spectra, have also been employed.

4.1.1. Aflatoxins.

Aflatoxins are considered among the most acutely hepatotoxic and carcinogenic mycotoxins affecting livestock, domestic animals and humans throughout the world. Accordingly, the legal limits in foods are set at trace levels in many countries, as for instance in the European Union and US, where the maximum values are 15 and 20 ng/g, respectively [82]. Given their neutral character, the separation of the different members of this group is possible by reversed-phase HPLC [81]. However, some drawbacks of these RP-HPLC methods, including long analysis times, complex gradient elution and large quantities of organic solvents, have encouraged the development of faster and less contaminant analytical procedures. Based on the ability to separate neutral and charged compounds, MEKC is an attractive alternative to conventional chromatographic methods for the analysis of aflatoxins. Using MEKC-LIF, the separation of aflatoxins AFB₂, AFG₁ and AFG₂ was

first achieved by Balchunas *et al.* in less than 25 minutes [83]. Authors pointed out that the addition of organic solvent to the separation buffer, containing 0.05 M sodium dodecyl sulfate (SDS), was necessary in order to obtain baseline separation of these compounds [83]. Faster separations of these three pollutants were demonstrated by Cole *et al.* [84] by adding 0.05 M sodium deoxycholate (DOC) to a background electrolyte free of organic solvents. Later, the separation of the three aflatoxins plus aflatoxin AFB1 by MEKC was achieved by the same research group [85]. Among the parameters examined, the use of capillaries with small internal diameter ($< 50 \mu\text{m}$) and low buffer concentrations were essential to achieve rapid separations at high voltages without critical losses in efficiency. The LOD of the method was estimated to be about $1 \mu\text{g/g}$ based on the analysis of artificially contaminated samples. Nevertheless, the presence of interferences prevented the identification of some analytes when real corn samples were analyzed.

Further improvements in MEKC sensitivity of the four aflatoxins (AFB1, AFB2, AFG1 and AFG2) in contaminated corn were provided by Maragos and Greer [86, 87]. Interestingly, aflatoxins exhibited enhanced fluorescence when dissolved in the BGE. The sensitivity of the method was also explored comparing two commonly used techniques for aflatoxins purification from corn samples. The analytes were purified from artificially contaminated corn using either a chloroform-based extraction procedure or an affinity column cleanup procedure and then, analyzed by MECK-LIF. The LOD achieved with the former extraction method was 0.5 ng/g , which was slightly better than the one obtained with the affinity column (i.e., 1 ng/g). However, authors highlighted the benefit of using the affinity column in terms of speed and simplicity. Correlation with an HPLC method was good, with a $R^2 = 0.969$ for 40 naturally contaminated samples [86]. In the same laboratory, the detection of the four mycotoxins was later selectively achieved using diode-array detection (DAD)

according to the distinctive UV spectra obtained for individual aflatoxins. However, this method is limited by a relatively large concentration of aflatoxin [88].

Dickens and Sepaniak [89] developed a separation-based fiber-optic sensor and its performance was tested for in situ monitoring of aflatoxins by MEKC. The instrument consisted of a modular device coupled to a capillary electrophoresis apparatus. The detection module was based on a He-Ne laser and a dual-fiber system designed for on-column detection, in which one fiber was the excitation fiber and the other was the emission-collection fiber. A refractive index matching gel was used in order to minimize background levels resulting from laser scatter at the capillary and fluorescence scatter generated in the excitation fiber. Although, four aflatoxins were baseline resolved and the LODs for these analytes in water were estimated to be lower than 10 pg/mL, the lack of adequate standard solutions prevented analyte quantification.

4.1.2. Fumonisin.

The isolation and characterization of fumonisins is relatively recent [90]. Fumonisin are a family of water-soluble toxins produced by different strains of *Fusarium verticillioides* (*moniliforme*) and *F. proliferatum*. Associated with several fatal diseases in animals and human esophageal cancer, fumonisins represent a serious concern for many countries. Given the widespread presence of these toxins in corn and their potential risk to public health, a number of separation methods have been developed for their detection and quantification in different matrices [91]. However, most of separation methods demand previous sample derivatization steps with fluorescent labels due to the molecular structure of fumonisins, which features four carboxylic acid moieties but lacks of a strong UV chromophore. Fumonisin type 1 (FB1) was analyzed by means of CE-MS [92]. Different

selectivities were obtained as a result of using both types, bare and coated fused-silica capillaries. The limit of detection for FB1 was close to 150 ng/g, however, the authors encountered sample precipitation problems during the analysis, which contributed to decrease the peak area precision and hampered the quantification of the toxin.

Two CE methods with fluorescence detection have been developed for the detection and quantification of FB1 in different matrices, including corn and animal feed. Maragos *et al.* [93] published an analytical procedure based on derivatization of fumonisins with fluorescein 5-isothiocyanate (FITC) previous CE-LIF analysis. In this study, the separation of FB1 from FB2, which differ by a single hydroxyl group, was achieved and coefficients of variation for the migration times lower than 3.5% were reported. A comparison between CE (with an argon ion LIF detector) and reversed-phase HPLC (with a standard fluorescence detector) in terms of sensitivity and waste generation demonstrated the potential of the CE-LIF method for the analysis of fumonisins, being about 50-fold more sensitive for the detection of FITC derivative of the toxins and 100-fold less contaminant than the HPLC method [93]. Authors reported a limit of detection of 50 ng/g of FB1 from corn [94]. Holocomb and Thompson [95, 96] used pre-column derivatization with 9-fluorenylmethoxycarbonyl (FMOC) and a less powerful fluorescence detector for the analysis of FB1 in different matrices. In this case, a C18 Sep-Pak Vac column was used for cleaning-up spiked corn and feed extracts. Recovery values from spiked feed and corn averaged 87 and 96% over a range of 2-20 µg/g and 1-20 µg/g, respectively. A LOD of 500 ng/g was reported from the analysis of FB1 in feed and corn. In addition, the quantitative estimations obtained from the CE analysis of samples with FB1 content ranging from 5 to 150 µg/ml were in good agreement

with data obtained from application of HPLC. However, for the analysis of the same set of samples, CE provided higher coefficients of variation than HPLC.

Later, Miyahara *et al.* [97] used a simple CE method together with an UV detector working at 185 nm, to detect underivatized samples. The method was applied to assay the purity of standard solutions containing fumonisins; nevertheless, it was observed to be 4000 times less sensitive than Maragos's method [93].

4.1.3. Ochratoxin A.

The presence of ochratoxin A (OA) in food and feed represents a serious concern worldwide since the exposure to this ubiquitous mycotoxin, produced by different species of *Aspergillus* and *Penicillium* fungi, has been related with human nephropathies and tumors of urinary organs [82]. Apart from a MEKC method reported by Holland and Sepaniak [98] (that will be discussed later in section 4.1.5), the analysis of OA has mainly been investigated by the most simple CE mode. Besides, owing to its good fluorescent properties, OA can be determined very sensitively using fluorescence detection. Thus, Corneli and Maragos [99] developed a CE-LIF method for the determination of OA in roasted coffee, corn and sorghum. In this study, the detection system consisted of a He-Cd laser (λ_{ex} 325 nm) and a set of filters allowing for the light collection between 400 and 539 nm. Using a sodium phosphate buffer adjusted at pH 7.0, separations were completed in 10 minutes and the LOD for OA was estimated to be 0.2 ng/g for all the matrices analyzed. To achieve such low LOD, the extraction of the analyte from the samples and the use of a tandem solid-phase extraction (SPE) cleanup procedure, which combined a silica column and an ochratoxin specific affinity column, were required. The reproducibility (given as %RSD) of migration times of

OA between 4 different days was 2.08% (n=17). Later, Calcutt *et al.* [100] adapted the method to determine the diffusion coefficient of OA in aqueous buffers using UV detection. Their findings brought some insights about the oxidative properties of the mycotoxin in biological systems.

González-Peñas *et al.* [101] recently reported a new CE method with diode array detection (DAD) for the analysis of OA in wine. In this work, the effect of various parameters on the separation efficiency and sensitivity was studied. Wine samples were first extracted with chloroform, cleaned-up using an immunoaffinity column and then analyzed by CE in less than 4 minutes, demonstrating the good possibilities of the method for quantitative purposes. Moreover, a comparative study between CE-DAD and HPLC with fluorescence detection showed a good correlation between both methods when nine fortified wine samples within the range 60-150 ng/mL were analyzed. Unfortunately, the limit of quantification for OA with CE-DAD method was 60 ng/mL, which is much higher than the permitted level for wine (2.0 ng/mL) in the EU.

4.1.4. Other mycotoxins.

Moniliformin is an acidic secondary metabolite produced by several *Fusarium* species. This mycotoxin has been associated with Keshan disease in humans, but no conclusive evidences have been reported yet [102]. Despite of this, the availability of sensitive methods for the analysis of moniliformin is desirable since this toxin has a negative impact on poultry industry. Maragos [103] developed a fast CE-UV method to detect moniliformin in corn. Using a purification procedure based on extraction with organic solvents and clean-up with a strong anion exchange column, 63 samples of maize were tested by an established HPLC-UV and by the proposed CE-DAD method.

Moniliformin could be detected within the range 0.2-5.0 µg/g and the LODs were estimated to be 0.05 µg/g and 0.1 µg/g for HPLC-UV and CE-DAD, respectively. The data obtained by both methods correlated well, although a slight underestimation by CE-DAD relative to HPLC-UV was reported. A number of benefits of using CE-DAD over HPLC-UV were discussed by the author, including a lower cost in reagents and sample, and a decreased analysis time, while a slightly lower sensitivity and a higher susceptibility to changes in the ionic strength of the sample would be some of the CE drawbacks indicated in that work [103].

Tsao and Zhou [104] reported the separation of patulin in 7 minutes by MEKC-DAD analysis. Despite it was not necessary either extensive extraction or cleanup procedures for good electrophoretic separation, analyte degradation problems were encountered during the method development process. The authors tested the method on apple cider samples and recovery percentages were higher than 91% with %RSD values between 4 and 16%. The proposed method was claimed to be sensitive enough to analyze patulin in apple cider because the LOD achieved (3.8 µg/L) was below the maximum limit (50 µg/L) established by many countries.

4.1.5. Multitoxin analysis.

As the limits allowed in foods for mycotoxins are strictly regulated, the development of fast and sensitive methods for the simultaneous separation and determination of several groups of mycotoxins is a very interesting topic. On the other hand, the variety of structural features and sample matrices for mycotoxins make this development extremely difficult. MEKC with UV detection plays an important role in multi-mycotoxin analysis. Holland and Sepaniak [98] developed an analytical procedure for the MEKC analysis of 10 mycotoxins in a single run. The

features of two different micellar systems, SDS and DOC, were investigated for the separation of neutral (zearalenone, roridin, sterigmatocystin and the four aflatoxins above mentioned, AFB1, AFB2, AFG1 and AFG2) and acidic (citrinin, penicillic acid and ochratoxin A) mycotoxins. Both buffer systems provided distinct selectivity for the separation of complex mycotoxin mixtures, mainly due to the differences in the properties of the two types of micelles. In order to improve the reproducibility, authors used normalized electrophoretic data from the separation of the 10 mycotoxins obtained under the two optimized buffer conditions (see Figure 5).

As stated before, the ubiquitous nature of mycotoxigenic fungi, and the multitude of toxins produced by some individual strains support the need for a broadly sensitive screening assay. Peña *et al.* [105] developed an analytical scheme which included a screening step for naturally fluorescent mycotoxins prior the confirmative step by MEKC. The screening system, which consisted of a flow injection analysis (FIA) system coupled to a spectrofluorimeter, provided data about the total content of aflatoxins in the sample. Subsequently, samples with total mycotoxin values above a certain threshold were subjected to MEKC-UV analysis for further identification. Samples with mycotoxin content close below the established threshold level were cleaned and pre-concentrated using a continuous flow system, including a C₁₈ minicolumn prior the confirmative MEKC analysis. Under optimized separation conditions, the reported limits of detection of four aflatoxins (AFB1, AFB2, AFG1 and AFG2) and two ochratoxins (OA and OB) ranged from 0.02 to 0.06 mg/L. Saving in time and costs are the benefits of this screening-confirmatory combination since MEKC analyses are preserved only to strictly necessary samples.

Recently, Martín *et al.* [106] explored the combination of MEKC and randomly amplified polymorphic DNA (RAPD) for the characterization of mycotoxigenic fungi species in contaminated food. Secondary metabolites from fungi isolated from dry-cured meat were analyzed by MEKC-DAD. Standard mycotoxins citrinin, penicillic acid, OA, cyclopiazonic acid, mycophenolic acid, zearalenone, AFB1, sterigmatocystin, patulin and griseofulvin were identified and most of them were found among the electrophoregrams of the fungi tested. The distinct peak profiles allowed the tested molds to be distinguished at species level, since most of the obtained secondary metabolites are different among the studied species. Moreover, characteristic RAPD patterns provided complementary data for the characterization of the mycotoxigenic fungi.

4.2. Marine and freshwater toxins.

A great number of marine bacteria and microalgae (including dinoflagellates, cyanobacteria and diatoms) are also responsible for the production of toxins [107]. Dinoflagellate and diatom toxins impact human health through the consumption of seafood while cyanobacteria toxins are mostly found in water. Certain herbivorous fish, zooplankton and filter-feeding shellfish become toxic after feeding on toxigenic microorganisms, and accumulating these toxins above a threshold level. Ingestion of toxic seafood can lead to five major human poisoning syndromes: paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), diarrhetic shellfish poisoning (DSP), ciguatera fish poisoning (CFP) and amnesic shellfish poisoning (ASP).

Some of the main drawbacks of the existing *in vivo* methods are that they cannot provide qualitative information concerning the nature of the toxin components in a complex mixture, tend to be less sensitive and precise when compared to instrumental analytical procedures, and are increasingly

unacceptable as methods of regulatory evaluation owing to ethical considerations associated with the use of live animals [108]. *In vitro* biological methods often feature excellent sensitivity but may be characterized by false-positive reactions and are only recently becoming available in commercial test kits appropriate to routine use. Owing to the chemical diversity of marine and freshwater toxins, there is no simple and rapid method to determine simultaneously various types of toxins from a single sample. As a result, there is a myriad of methods developed to detect a single compound or a group of related toxins. Among the instrumental methods, HPLC has been the most widely used to study individual marine and freshwater toxins in complex mixtures. However, the drawbacks found using HPLC (*vide infra*) have made necessary the development of new analytical methods including those based on CE techniques.

4.2.1. PSP toxins.

PSP neurotoxins are a family of complex organic compounds known as saxitoxins (STXs), the related compound tetrodotoxin (TTX) and their analogues. The basic structures of PSP neurotoxins are composed of a 3,4-propinoperhydropurine tricyclic system containing guanidinium groups [109]. More than 20 derivatives of saxitoxin with different toxicity have been described to occur naturally in phytoplankton and shellfish [110]. Detection of PSP toxins by HPLC has represented a significant challenge due to their highly polar nature and the lack of useful UV chromophores absorbing above 220 nm. In consequence, a number of sensitive HPLC methods using ion-pairing and post-column reaction permitting fluorescence detection have been developed providing LOD as low as 0.3 ng/mL for STX [108]. However, the sensitivity of these methods is dependent on many parameters (reagent concentration, reaction time, pH and temperature of the post-column reaction), and the formation of the oxidation products as well as the conversion of different PSP toxins into

common fluorescent derivatives prevents, in some cases, the unambiguous determination of toxin profiles [108].

The presence of readily ionizable groups and their volatile nature makes these PSP toxins perfect candidates not only for CE but also for MS ionization techniques such as ESI. Nevertheless, initial attempts to determine PSP by CE included the use of LIF detection and pre-column derivatization of the toxins with different reagents [111]. Problems associated with degradation of derivatized saxitoxins suggested that sensitive and selective instrumental methods enabling identification of native PSP toxins would be preferable. Thus, Thibault *et al.* [112] explored the capabilities of CE-UV (using detection at 200 nm) for the analysis of native PSP toxins in dinoflagellate and shellfish extracts. Toxins were extracted from algal cells and scallops livers and then, purified using a Biogel P-2 column followed by CE-UV analysis. Good resolution and sensitivity in the separation of STX and neosaxitoxin (NEO) were achieved using a 20 mM solution of sodium citrate adjusted to pH 2.1. Under the conditions assayed, good linearity (correlation coefficient = 0.998) over the concentration range 1.5-300 µg/mL was attained. However, the method provided poor sensitivity (concentrations higher than 5.0 µM of STX could only be detected) when it was compared with mouse bioassay (0.5 µM), HPLC with fluorescence detection (0.01 µM) and flow-injection ionspray- MS (0.1 µM). In addition to STX and NEO, other PSP toxins were detected, namely, gonyautoxins (GTX2 and GTX3), but C toxins, which have no net charge at pH 2.1 were not separated [112].

The potential of CE-MS for the confirmation of UV peak identities was also explored [112, 113]. In this preliminary study [112], a CE-MS interface, which consisted of a modified commercial

IonSpray probe on a coaxial arrangement was employed. This interface was used to couple the CE instrument to an API III triple quadrupole mass spectrometer. A limit of detection of 100 pg of STX was reported. Pleasance *et al.* [113] made further improvements to the CE-MS system in order to compare the ease of use, sensitivity and electrophoretic performance of two types of interfaces for the CE-MS coupling. Namely, a liquid-junction and a coaxial arrangement were examined using an API III triple quadrupole mass spectrometer and three commercially available capillary electrophoresis systems, which allowed the study of the effect of different injection techniques on the linearity response of the detector. Pressure injection provided the best results over electrokinetic and split-flow injection techniques in terms of reproducibility and quantitative performance. Although both interface designs provided efficient CE-MS coupling, the coaxial configuration offered more robustness and better reproducibility. A limit of detection of 10 pg of STX was achieved using the coaxial configuration together with pressure injection. Although other toxins, including okadaic and domoic acid, were identified by this methodology, the insufficient sensitivity of the method precluded its application for routine analysis at the demanded range.

Further developments on CE methods were focused towards improving the concentration detection limits for toxins. In order to achieve this, capillary isotachopheresis (CITP) was used as pre-concentration technique prior to CE, with both UV and MS detection, for the analysis of PSP toxins in shellfish tissues [114]. Different leading electrolytes, including β -alanine, triethylamine, and morpholine were evaluated regarding both aspects, their applicability to the preconcentration process and their compatibility with the electrospray process taking place prior to MS analysis. In a 75 μm i.d. x 107 cm polyacrylamide-coated capillary, effective stacking was achieved with an injection volume of 2.2 μL of a sample dissolved in acetic acid; 35 mM morpholine/formate buffer

at pH 5 as leading electrolyte; and 10 mM formic acid as terminating electrolyte. The CITP/CE-UV method demonstrated good quantitative performance for the STX, NEO and GTX2,3 toxins providing good linearity ($R^2=0.998$) over a wide concentration range (10^{-8} - 10^{-4} M) and the LODs were determined as 10 nM for STX and NEO and 20 nM for GTX2,3. For CE-MS analysis of PSP toxin standards and extracts from contaminated shellfish (see an example in Figure 6), lower limits of detection were obtained using polyacrylamide-coated capillaries of 50 μ m i.d. than using 75 and 100 μ m i.d. capillaries. This was attributed to the higher separation currents and background chemical noise associated with the latter columns. Analyses performed in selected ion monitoring (SIM) mode provided LODs of 16 nM for STX and NEO and 30 nM for GTX2,3 toxins, which is an improvement in detection limits of 2 orders of magnitude with respect to previous studies. The applicability of this methodology to the analysis of real samples was corroborated by Gago-Martinez *et al.* [115]. In this study, samples were analyzed by two methodologies, CITP/CE-MS using the SIM acquisition mode and HPLC with fluorescence detection and post-column derivatization. The results obtained by HPLC were unsatisfactory due to the difficulty for identifying most of the peaks in the chromatograms. In contrast, CITP/CE-MS revealed the presence of decarbamoyl derivatives of STX, GTX2 and GTX3. In view of the risk of a reduction in the separation performance due to irreversible modification of the capillary coating when a large number of crude sample extracts have to be analyzed, the pre-concentration CITP/CE method was modified to feature the use of bare-fused capillary columns [116]. The method was also tested with MS detectors in both, CE-MS and CE-MS/MS configurations, to study the enzymatic production of carbamoyl derivatives of the following PSP toxins: STX, NEO, GTX1,2,3,4, C1, C2 and B1 in shellfish extracts [116].

Piñeiro *et al.* [117] used a 75 μm i.d. x 104 cm polyvinylalcohol-coated capillary under similar conditions to those used in the method reported by Locke and Thibault [114], except that in this occasion DAD was employed as detector. LODs of 0.5 and 0.06 $\mu\text{g/mL}$ were calculated for STX and dcSTX, respectively. However, a lower resolution in the GTX group was observed when real contaminated mussel samples were analyzed, results that may be associated with the complexity of the matrix, its high salt content and the presence of interferences.

Quantitative aspects of the application of CITP-CE to the analysis of a wider set of PSP toxins have been addressed in a recent paper by Wu *et al.* [118]. The effect of five experimental conditions (including time and voltage during CITP process, pH and concentration of the BGE, and separation voltage) on the efficiency and resolution of the separation was investigated. The method provided linear responses ranges from 1.4 to 200 mM toxin and the LODs (S/N=3) were between 0.1 and 0.3 μM depending on the analyzed toxin.

4.2.2. ASP toxins (domoic acid).

The water-soluble tricarboxylic amino acid, known as domoic acid (DA), is the recognized toxic agent of ASP and the only shellfish poison produced by a diatom. In addition, plankton and shellfish samples may contain small amounts of isomers (isodomoic acids) following exposure to UV light [107].

Initial work by Nguyen *et al.* [119] on developing a CE-UV method to detect DA in mussels confirmed the potential of the technique for the analysis of the toxin. Apart from some difficulties linked to capillary wall regeneration (and, therefore, for obtaining reproducible migration times) the

LOD was below the regulatory level for this toxin (20 ppm). Later, Zhao *et al.* [120] developed a CE-UV method with enhanced selectivity in detecting DA and four isodomoic acids in seafood. To achieve this, crude homogenates were subjected to a tandem SPE cleanup step, combining both, strong anion-exchange and strong cation-exchange columns. The clean extract was then injected in a CE-UV system operating at 242 nm. Analytes were effectively resolved using a BGE containing 22.5 mM sodium tetraborate and 20 mM β -cyclodextrin. This approach provided a better resolution of the isomers than did HPLC and its applicability to real samples was demonstrated in mussels, razor clams and anchovies. As low as 3 pg of DA could be detected, but, as expected, CE was shown to be less sensitive than HPLC for shellfish samples.

Piñeiro *et al.* [117] used a sample clean-up procedure based on the tandem SPE procedure previously developed by Zhao *et al.* [120] with some modifications, and studied the effect of different BGEs on the separation of DA in razor clams and mussels by CE-UV. A mean recovery value of 103% and RSD values of 0.158% and 0.582% for migration times and areas, respectively, were reported [117]. This method has recently been applied to the analysis of certified standard mussel material containing DA, as well as naturally contaminated samples [121]. In spite of the worse sensitivity of the CE method compared to HPLC, the sensitivity of CE was sufficient to determine the amount of DA present in real samples under the regulatory levels.

Also, the detection of DA has been investigated by means of capillary electrochromatography (CEC) with UV detection [122]. Authors presented preliminary results on the analysis of contaminated razor clams obtained using a capillary column of 75 μm x 20 cm, packed with 3- μm C18-bonded silica particles and a mobile phase containing 40% of 5 mM phosphate buffer at pH 2.5

and 60% acetonitrile. Effective resolution was achieved, but the sensitivity of the CEC-UV method resulted unsatisfactory to monitor DA at regulatory levels in seafood. Further improvements of this method including the use of a capillary column with higher internal diameter, and the adjustments of other parameters such as injection time and voltage allowed for a better sensitivity. However, the lack of robustness and reproducibility were reported by the authors as the main drawbacks of this CEC method [121].

4.2.3. DSP toxins (okadaic acid).

Okadaic acid (OkA) and its analogues (DTX-1, 2 and 3) are known as causative agents of DSP syndrome and represent a potentially threat to human health as potent tumor promoters. Boland *et al.* [123] used CE with UV detection at 200 nm for the analysis of OkA in semi-purified mussel extract that had previously been screened using HPLC and protein phosphate assays. The addition of SDS to the separation buffer allowed OkA to be effectively separated from the other compounds. Later, this MEKC-UV method was applied to the determination of DSP toxins in shellfish extracts. The micellar phase was composed of 12.5 mM borate buffer at pH 9.2 and 20 mM SDS. A LOD of 40 pg was reported and it would allow for the detection of OkA at concentrations below the regulatory levels in shellfish in most of the countries [124]. Li *et al.* [125] improved the MEKC-UV method in order to determine OkA and DTX-1 in three kinds of shellfish. The micellar phase containing a higher concentration of SDS (40 mM) and 10% methanol allowed the effective resolution of both compounds. The applicability of the method to real samples was demonstrated by a survey on the distribution of DSP toxins in a particular sea area of China [125].

4.2.4. Microcystins and other cyanobacterial toxins.

Microcystins (MC) are cyanobacterial toxins that impact human health mainly through drinking water contamination [110]. About 60 variants of MC have been characterized so far being recognized as hepatotoxic and tumor-promoting compounds. These compounds are cyclic heptapeptides and because of their low absorption under UV, an extensive sample clean-up treatment is needed in order to detect them by chemical methods [126]. Bouaicha *et al.* [127] developed a MEKC-UV method for the detection of three microcystins (MC-YR, MC-LR and MC-RR) in cyanobacterial extracts. The incorporation of 10 mM SDS to the BGE was a key factor in order to achieve effective resolution of the three MCs. Good linearity was reported in the concentration range 2.5-50 µg/mL, the mass detection limit was ~7.5 pg for each toxin. Nevertheless, a SPE clean-up step appeared to be an essential requirement for the detection of these toxins in cyanobacterial extracts.

Gago-Martínez *et al.* [121] explored the potential of using immunoaffinity cartridges for selective extraction of MCs and field-amplified sample stacking for on-line preconcentration of the toxins during the MEKC analysis of water. The presence of MC-RR and MC-LR in the extracts was confirmed by means of CE-MS [121].

4.2.5. Other toxins.

Brevetoxin, one of the ethiological agents of NSP syndrome has been analyzed by MEKC-LIF [128, 129]. The micellar phase consisted of 10 nM sodium borate buffer and 100 mM cholate at pH 9.3. Sample was injected into the capillary by pressure and the LOD calculated with a standard solution was 25 fg, while a value of 200 fg was obtained when using a seafood extract. This

difference was supposed to be due to the higher background fluorescence of the matrix in the extract.

Bouaicha *et al* [124] investigated the use of CE-UV for the detection of maitotoxin (MTX), a potent poison produced by a dinoflagellate, which is transmitted through the food chain to the viscera of some fishes. For the analysis, a polyvinylalcohol-coated fused silica capillary column was used in order to prevent the adsorption of the MTX onto the silica wall. Owing to the lack of a pure calibration standard, the identity of the electrophoretic peak of MTX was confirmed by in vitro cytotoxicity assay. The detection limit was found to be 50 pg of MTX, pointing out the potential of this CE-UV technique for the detection of low levels of the toxin.

5. Concluding remarks and future outlooks.

Despite the remarkable advances on microbiological food analysis by CE shown in this review, the development of new CE separation and detection strategies applicable to real-life food analysis will keep growing in order to comply with the everyday more and more strict regulations. It is expected that some of the limitations of current CE methods, mainly, sensitivity, will be improved by different means (more powerful detection schemes, microbial capture techniques applicable to a wide variety of matrices, etc.). In this regard, the development of new strategies aimed to concentrate microbial cells while keeping them intact prior to their CE analysis will be a hot topic in the future. In this context, apart of improving classical centrifugation and filtration, the use of more selective microbial capture techniques such as immunomagnetic separation and immobilized recognition molecules, will be interesting candidates to reach this goal. On the other hand, the development of more CE methods based on MS detection to confirm the presence of microbial

contaminants is predictable. It is also expected an increasingly growing development of microchips to provide fast analysis in-situ, allowing to trace the origin of the contamination and subsequently, to quickly reduce the risk of food poisoning. Also, there is an important demand of suitable standards to assist with the method development process.

Acknowledgements

V.G-C. would like to thank CSIC for a I3P postdoctoral grant. This study has been supported by an AGL2005-05320-C02-01 Project (Ministerio de Educacion y Ciencia) and the S-505/AGR-0153 ALIBIRD-Project (Comunidad Autonoma de Madrid).

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Table 1. Applications of CE to determine microbial contaminants.

Microbial contaminant or toxin analyzed	Sample	CE conditions	Ref.
<i>Staphylococcus aureus</i> , <i>Enterococcus faecalis</i> , <i>Streptococcus pyogenes</i> , <i>Streptococcus agalactiae</i> ,	Culture broth	CZE-UV (190 nm); Buffer: 4.5 mM TRIS, 4.5 mM boric acid, 0.1 mM EDTA, buffer; Capillary: 250 cm x 100 μ m i.d.	[28]
<i>Escherichia coli</i> , <i>Pseudomonas fluorescens</i> , <i>Bifidobacterium longum</i> , <i>Paracoccus denitrificans</i>	Culture broth	CZE-DAD; Buffer: 10 mM phosphate; Capillary: 80 cm x 50 μ m i.d.	[32]
<i>S. aureus</i> , <i>Pseudomonas</i> spp., <i>Enterobacter aerogenes</i> , <i>Micrococcus luteus</i> , <i>Serratia rubidae</i> , <i>Saccharomyces cerevisiae</i>	Culture broth	CZE-UV (214 nm); Buffer: ~0.6 mM TRIS, 0.6 mM boric acid, 0.012 mM EDTA, 0.0125% PEO; Capillary: 27 cm x 100 μ m i.d.	[34]
<i>Bifidobacterium infantis</i> , <i>Lactobacillus acidophilus</i>	Dietary supplement	CZE-LIF (λ_{ex} 488 nm); Buffer: ~0.6 mM TRIS, 0.6 mM boric acid, 0.012 mM EDTA, 0.0125% PEO; Capillary: 27 cm x 100 μ m i.d.; Sample incubated with propidium iodide and SYTO-9	[37]
<i>Salmonella enteritidis</i>	Culture broth	CZE-LIF (λ_{ex} 488 nm); Buffer: 0.1 M TRIS, 0.1 M boric acid, 2 mM EDTA, 0.01% sodium alginate, 0.1% NaCl; Capillary: 31 cm x 75 μ m i.d.; Sample incubated with fluorescein-labeled antibodies	[44]
<i>E. coli</i> , <i>Proteus vulgaris</i> , <i>Bacillus megaterium</i> , <i>Arthrobacter globiformis</i> , <i>Micrococcus</i> spp.	Culture broth	CZE-UV (210 nm); Buffer: ~0.6 mM TRIS, 0.6 mM boric acid, 0.012 mM EDTA, 0.0125% PEO; Coated capillaries: 33.5 cm x 75 μ m i.d.	[52]
<i>E. coli</i> , <i>Salmonella</i>	Meat	CZE-LIF (λ_{ex} 488 nm); Buffer: 4.5 mM TRIS, 4.5 mM boric acid, 0.1 mM EDTA, 0.1% poly-Duramide; Coated capillaries: 27 cm x 75 μ m i.d. Sample incubated with fluorescein-labeled antibodies	[53]
<i>S. aureus</i>	Culture broth, feces	CZE-DAD. Buffer: ~0.6 mM TRIS, 0.6 mM boric acid, 0.012 mM EDTA, 0.0125% PEO; Sample incubated with antibody-coated latex particles.	[54]
<i>Yersinia enterocolitica</i> , <i>Leuconostoc mesenteroides</i> , <i>S. enteritidis</i> , <i>Listeria monocytogenes</i> , <i>E. coli</i> , <i>Lactobacillus plantarum</i> , <i>S.</i>	Juice, milk, corn flakes, baby food, frankfurter	CZE-DAD. Buffer: 25 mM phosphate, 25 μ M calcium chloride, 35 μ M myoinositol hexakisphosphate; Capillary: 39.5 cm x 75 μ m)	[55]

<i>aureus</i> , <i>Enterococcus faecium</i>			
<i>Clostridium botulinum</i>	Smoked fish	PCR-CGE-UV (260 nm); Coated capillary: 60 cm x 750 µm)	[66]
<i>Flavobacterium psychrophilum</i>	Fish	T-RFLP/CGE-LIF	[67]
<i>S. enterica</i> , <i>Campylobacter jejuni</i>	Culture broth	AFLP/CGE	[68]
Enterohemorrhagic <i>E. coli</i> O157:H7	Culture broth	PCR-CGE-LIF (λex 488 nm); Buffer: Tris-borate, thiazole dye; Coated capillary: 37 cm x 100 µm)	[70]
<i>Salmonella</i> , <i>L. monocytogenes</i> , <i>S. aureus</i>	Beaf meat	Multiplex PCR/CGE-LIF (λex 488 nm); 20 mM TRIS, 10 mM phosphoric acid, 2 mM EDTA, 1.5 M urea, 500 nM YOPRO1, 4.5% HEC; Capillary: 60 cm x 75 µm	[72]
<i>Leuconostoc</i> , <i>Carnobacterium divergens</i> , <i>C. gallinarum</i> , <i>C. maltaromicum</i>	Culture broth	Multiplex PCR/CGE-LIF (λex 488 nm) 20 mM TRIS, 10 mM phosphoric acid, 2 mM EDTA, 1.5 M urea, 500 nM YOPRO1, 4.5% HEC; Capillary: 60 cm x 75 µm)	[73]
<i>P. aeruginosa</i> , <i>S. Aureus</i> , <i>Straphylococcus epidermidis</i>	Culture broth	CZE-MS/MS of peptides; Buffer: 10 mM ammonium acetate, 10 mM acetic acid; Coated capillary: 100 cm x 75 µm i.d.	[78]
Aflatoxins AFB1, AFB2, AFG1, AFG2	Corn	MEKC-LIF (λex 325 nm); Buffer: 60 mM sodium deoxycholate, 6 mM sodium borate, 10 mM dibasic sodium phosphate	[86]
Fumonisin FB1 and FB2	Corn	CZE-LIF (λex 488 nm); Buffer: 7% methanol (v/v) in 50 mM borate buffer. Capillary: 50 cm x 75 µm i.d. Sample derivatized with FITC	[94]
Ochratoxin A	Roasted coffee, corn, sorghum	CZE-LIF (λex 325 nm); Buffer: 20 mM sodium phosphate; Capillary: 50 cm x 75 µm i.d.	[99]
Ochratoxin A	Wine	CZE-UV (380 nm); Buffer: 10 mM sodium tetraborate; Capillary: 40 cm x 50 µm i.d.	[101]
Moniliformin	Corn	CZE-DAD; Buffer: 150 mM dihydrogen phosphate; Capillary: 50 cm x 75 µm i.d.	[103]
Patulin	Apple cider	MEKC-DAD; Buffer: 25 mM borate, 50 mM SDS; Capillary: 50 cm x 75 µm i.d.	[104]
PSP toxins	Oysters	CZE-MS ; Buffer : Trisma buffer; Capillary: 90 cm x 50 µm i.d.	[113]
PSP toxins	Scalops	CITP-CZE-MS; Terminating electrolyte: 10 mM formic acid; Buffer: 35 mM morpholine; Capillary: 57 cm x 50 µm i.d.	[114]
ASP toxins (domoic acid, isodomoic acids)	Mussels, razor	CZE-UV (242 nm) Buffer: 22.5 mM sodium tetraborate and 20 mM β-cyclodextrin;	[120]

	clams, anchovies	Capillary: 75 cm x 50 μ m i.d.	
DSP toxins (okadaic acid, DTX-1, 2 and 3)	Shellfish	MEKC-UV (200 nm); Buffer: 12.5 mM borate, 20 mM SDS; Capillary: 64.5 cm x 50 μ m i.d.	[124]
Microcystins (MC-YR, MC-LR, MC-RR)	Marine extracts	MEKC-UV (200 nm); Buffer: 40 mM phosphate buffer, 10 mM SDS; Capillary: 64.5 cm x 50 μ m i.d.	[127]
Brevetoxin	Marine extracts	MEKC-LIF (325); Buffer: 10 mM sodium borate, 100 mM cholate; Capillary: 50 cm x 75 μ m i.d.	[128]
Maitotoxin	Algal extracts	CZE-UV (195); Buffer: 40 mM phosphate; Coated capillary: 50 cm x 50 μ m i.d.	[124]

FIGURE LEGENDS

Figure 1. CE-UV electropherogram of eight contaminating bacteria. Peaks: 1, *Y. enterocolitica*; 2, *L. mesenteroides*; 3, *S. enteritidis*; 4, *L. monocitogenes*; 5, *E. coli*; 6, *L. plantarum*; 7, *S. aureus*; 8, *E. faecium*. Redrawn from [55].

Figure 2. CE-LIF electropherogram of microorganisms at concentration of cells per mL of 8×10^4 *S. epidermidis*, 6×10^4 *E. faecalis*, 8×10^4 *E. coli*, 5×10^4 *C. albicans*. Time in x-axis till 30 min. Redrawn from [56].

Figure 3. CE-LIF electropherograms of single cells of various bacteria and fungi. Redrawn from [58].

Figure 4. CGE-LIF vs. slab agarose gel analysis of multiplex PCR reactions of inoculated raw beef samples with 5.7×10^2 , 7.9×10^2 , 2.6×10^3 cfu ml⁻¹ of *L. monocytogenes* CECT 4031^T, *S. paratyphi* CECT 554 and *S. aureus* CECT 435, respectively, incubated at different times. Samples: A) sample at 0 h incubation time; B) sample at 6 h incubation time; M) 100 bp ladder molecular weight standard (Pharmacia). (Sp) 163 bp *S. paratyphi* DNA, (Lm) 234 bp *L. monocytogenes* DNA and (Sa) 270 bp *S. aureus* DNA fragments. Redrawn from [72].

Figure 5. Electropherograms of the mycotoxins: a, citrinin; b, penicillic acid; c, OA; d, AFG2; e, zearalenone; f, AFG1; g, AFB2; h, AFB1; i, roridin A; j, Sterigmatocystin. (Top) SDS-buffer. (Bottom) NaDC-buffer. Redrawn from [98].

Figure 6. Analysis of a contaminated scallop liver extract using CITP/CZE-MS. TIC profile for m/z 220-500 obtained under full mass scan acquisition (A); extracted ion current profile for ΣMH^+ Ions at m/z 300, 316, and 396 (B) extracted from the analysis shown in (A). CITP/CZE-MS/MS using MRM acquisition mode (C), for the reaction channels m/z 300 - 204, 282 (10-20 min) and m/z 396 - 298, 316 (25-30 min). Redrawn from [114].

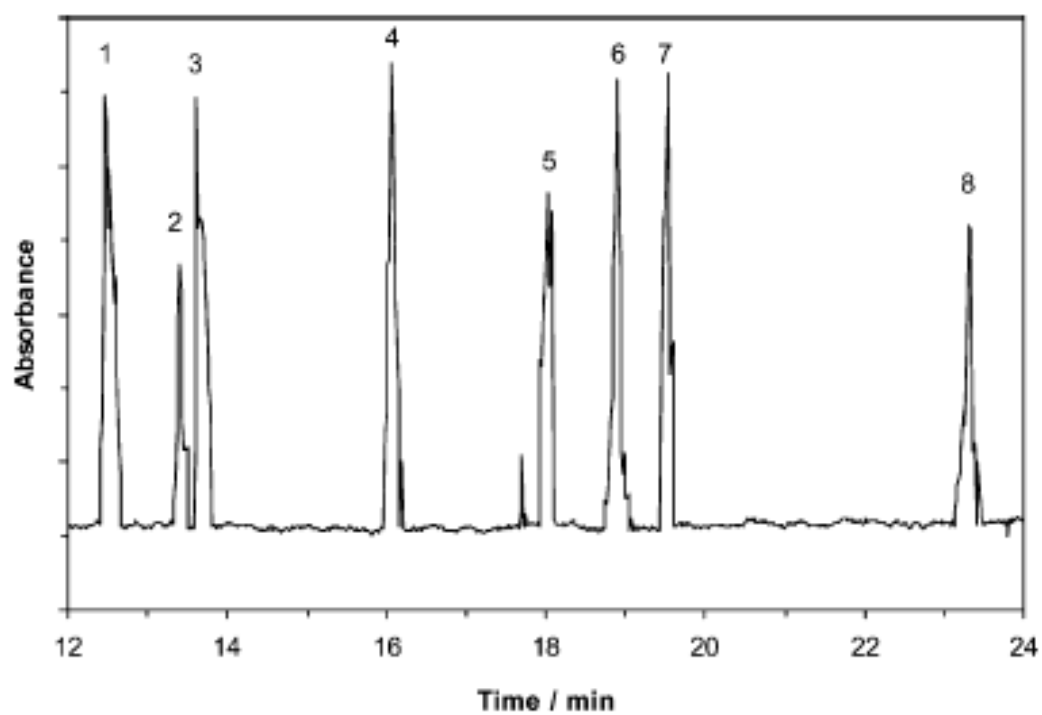


Figure 1

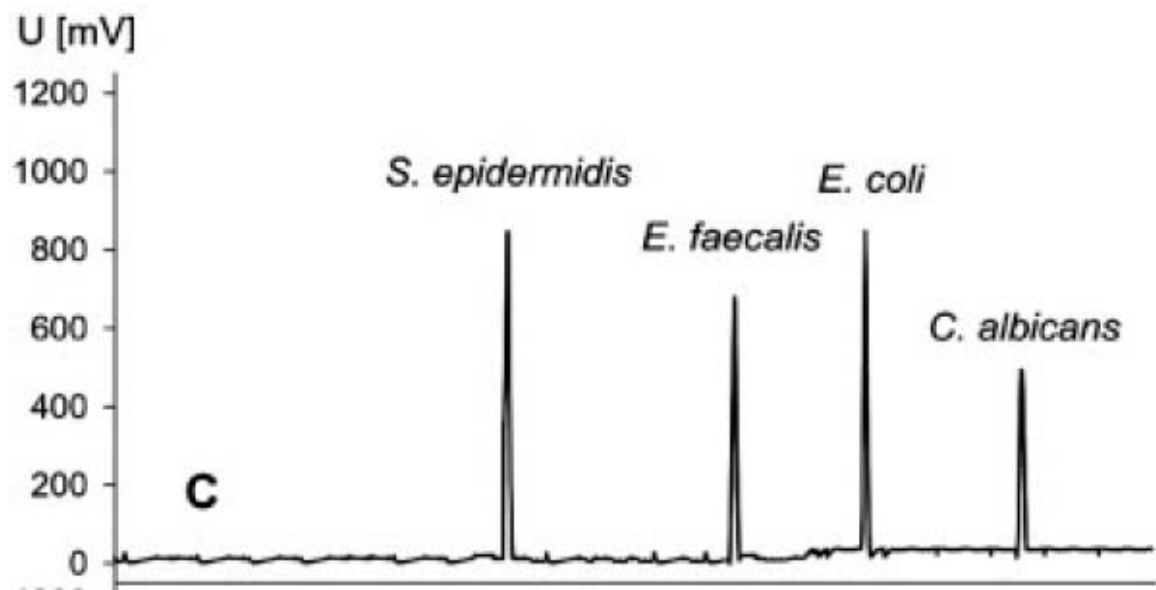


Figure 2

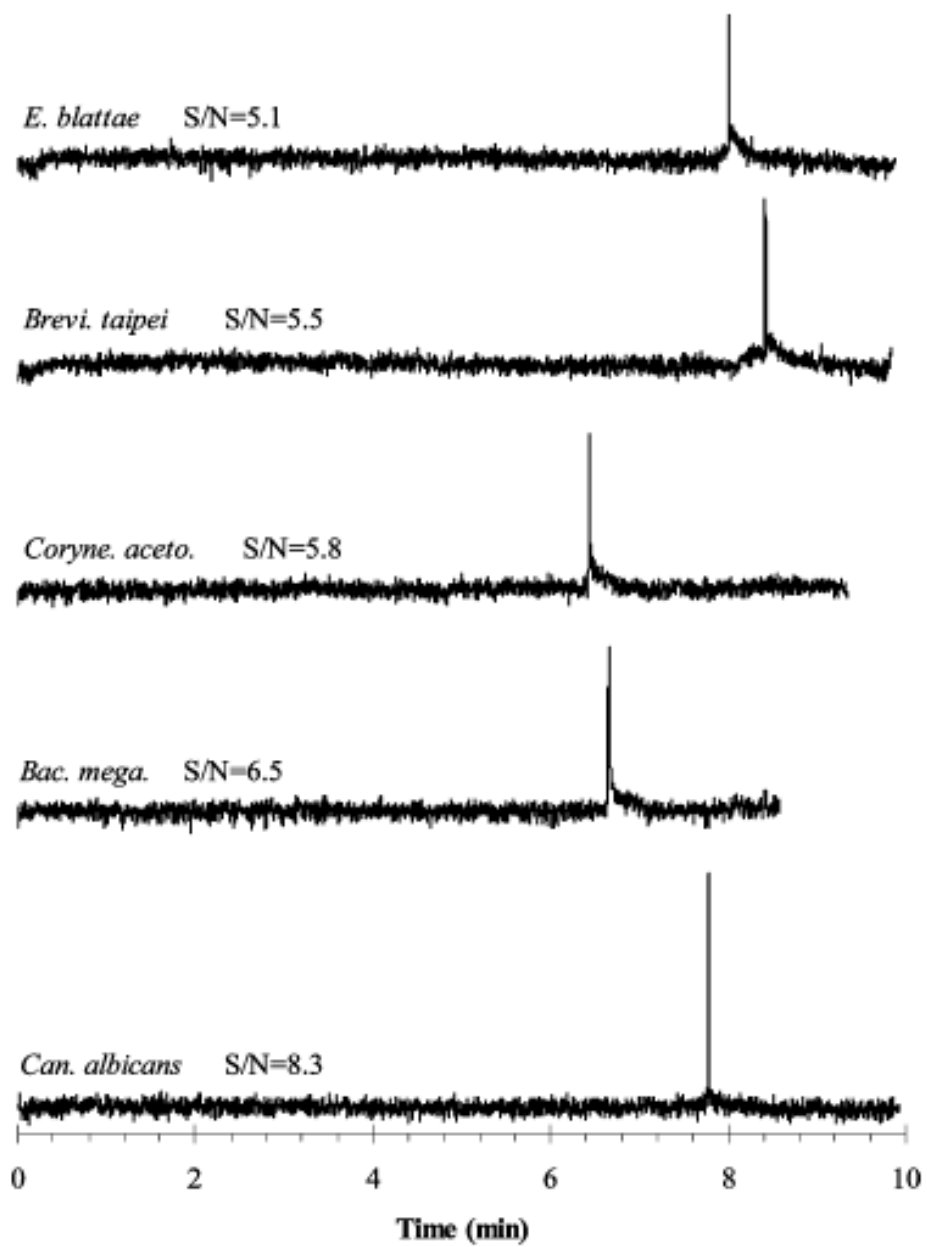


Figure 3

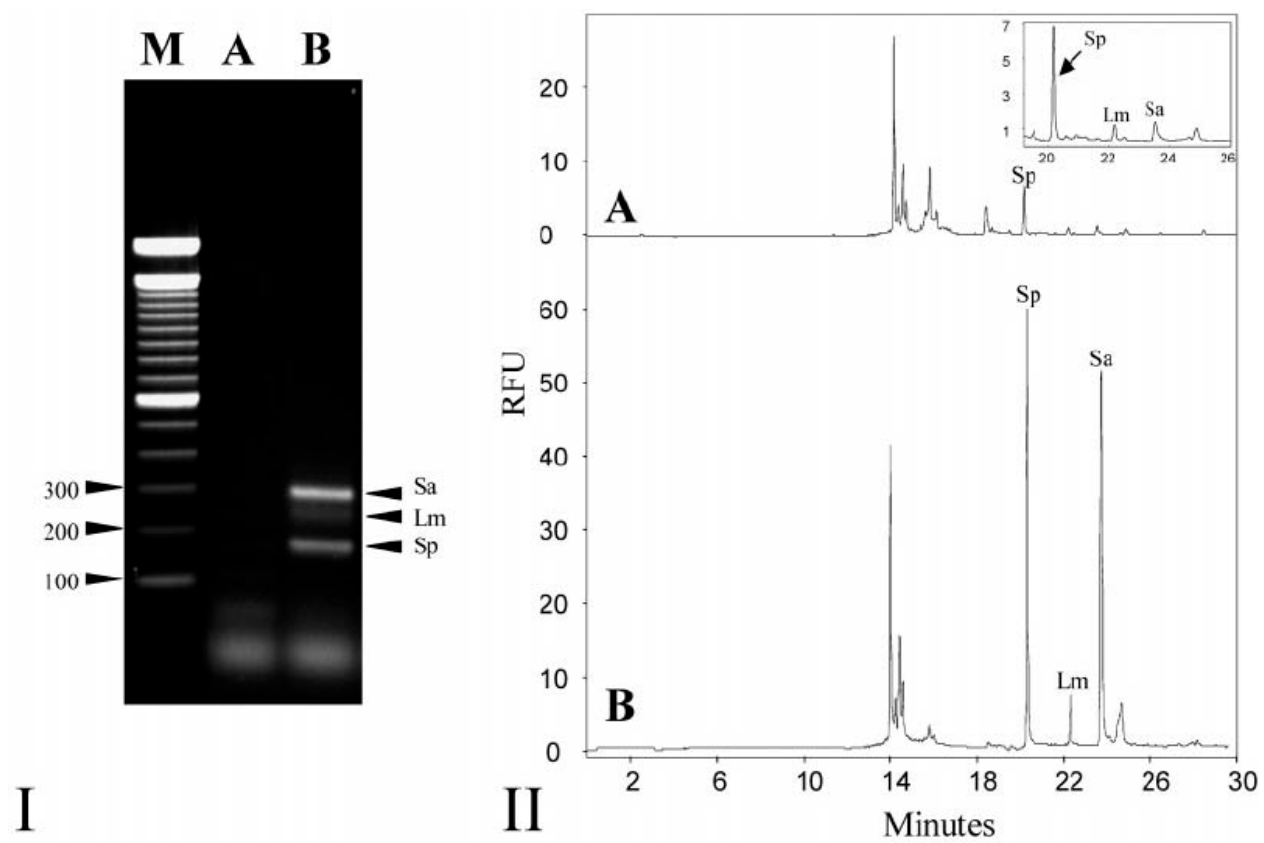


Figure 4

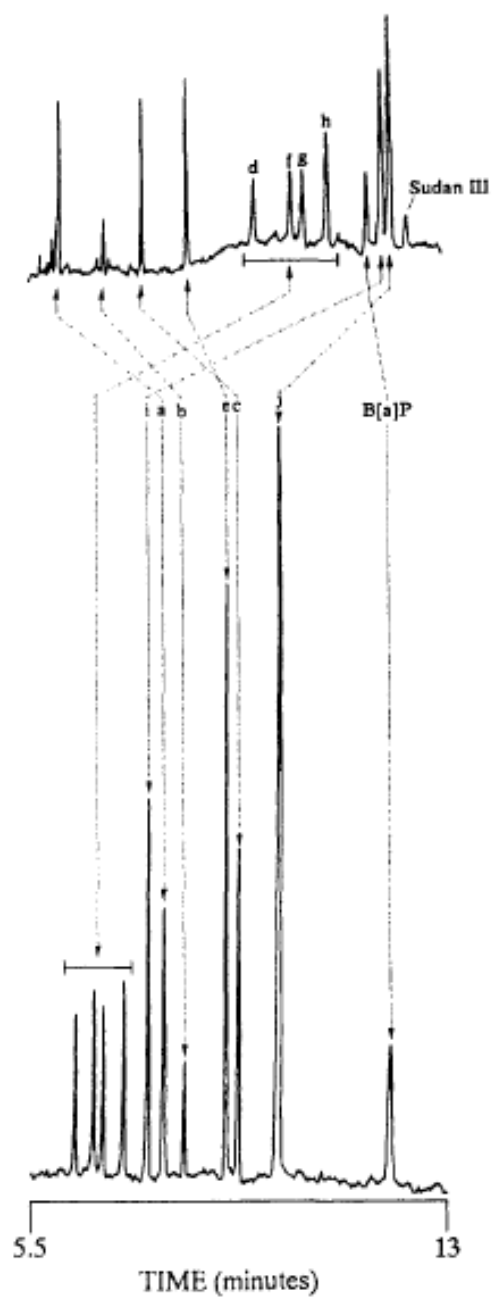


Figure 5

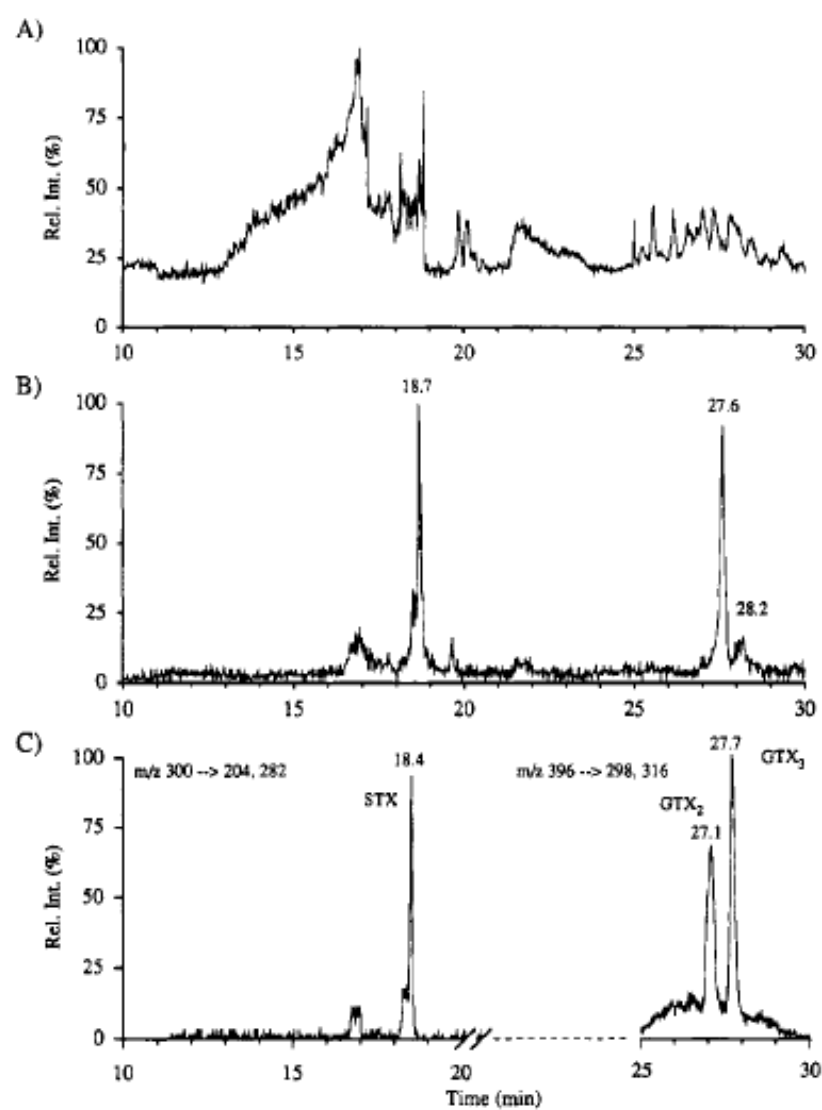


Figure 6